

Characterization of Inorganic Biocarriers That Moderate System Upsets during Fixed-Film Biotreatment Processes

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Inorganic matrices were developed for fixed-film bioreactors affording protection to microorganisms and preventing loss of bioreactor productivity during system upsets. These biocarriers, designated Type-Z, contain ion-exchange properties and possess high porosity and a high level of surface area, which provide a suitable medium for microbial colonization. Viable cell populations of 10^9 /g were attainable, and scanning electron micrographs revealed extensive external colonization and moderate internal colonization with aerobic microorganisms. Laboratory-scale bioreactors were established with various biocarriers and colonized with *Pseudomonas aeruginosa*, and comparative studies were performed. The data indicated that bioreactors containing the Type-Z biocarriers were more proficient at removing phenol (1,000 ppm) than bioreactors established with Flexirings (plastic) and Celite R635 (diatomaceous earth) biocarriers. More significantly, these biocarriers were shown to moderate system upsets that affect operation of full-scale biotreatment processes. For example, subjecting the Type-Z bioreactor to an influent phenol feed at pH 2 for periods of 24 h did not decrease the effluent pH or reactor performance. In contrast, bioreactors containing either Celite or Flexirings demonstrated an effluent pH drop to ~2.5 and a reduction in reactor performance by 75 to 82%. The Celite reactor recovered after 5 days, whereas the bioreactors containing Flexirings did not recover. Similar advantages were noted during either nutrient or oxygen deprivation experiments as well as alkali and organic system shocks. The available data suggest that Type-Z biocarriers represent an immobilization medium that provides an amenable environment for microbial growth and has the potential for improving the reliability of fixed-film biotreatment processes.

Immobilized-cell bioreactor technology provides a cost-effective means for the treatment of existing environmental waste problems, such as contaminated groundwater, or for the eradication of pollutants at their point of origin (7, 8, 11, 18, 21). This technology generally involves the colonization of a specialized microorganism onto inorganic biocarriers as fixed films and the utilization of these colonized surfaces in controllable reaction vessels or bioreactors. Bioreactors can be operated in either a packed-bed (fixed-bed) or a fluidized-bed configuration. Immobilized-cell bioreactor systems offer several advantages over conventional suspended-growth systems. For example, this technology favors high-concentration chemical loadings without washout, improved system productivity, less susceptibility to hydraulic and process upsets, and reduced sludge production (6, 19).

Ideally, suitable biocarriers for immobilization of microorganisms should be nontoxic and should provide a rough, irregular surface. The matrix should be hydrophilic (23) and porous (17); these properties have been shown by others to promote the adherence and proliferation of microorganisms (2, 12, 14–16, 19). A porous structure permits internal colonization that serves as a biomass reserve for recolonization of bioreactors after severe system shocks (9–11). Biocarriers commonly used for immobilization provide adequate surfaces for microbial adsorption but do little to protect or maintain the productivity of bioreactors under adverse conditions commonly encountered during full-scale biotreatment of aqueous waste streams.

In this communication, we describe a new biocarrier, Type-Z,

that meets the aforementioned criteria and therefore provides an excellent substratum for microbial colonization. In addition, this biocarrier, unlike existing products, is engineered to moderate process shocks. Comparative data evaluating the response of phenol-degrading pseudomonads immobilized onto various biocarriers during exposure to system shocks are presented. The results favor the conclusion that the Type-Z biocarrier is superior to existing products and that microorganisms colonized onto this surface may increase the dependability of immobilized-cell biotreatment processes.

MATERIALS AND METHODS

Microorganisms. The microorganisms used in this study were kindly provided by R. Portier (Louisiana State University). *Pseudomonas aeruginosa* 977 and 978 were maintained on Trypticase soy agar (Difco) or stored at -80°C in 10% glycerol-Trypticase soy broth.

Biocarriers. Biocarriers used for microbial immobilization included Grace Type-Z biocarrier (1/8-in. [1 in. = 2.54 cm]), Celite R635 (1/4-in. diatomaceous earth pellets; Johns Manville), and plastic 3/8-in. rings (Flexirings; Koch Engineering).

The internal microporous surface area of Type-Z biocarrier was determined by the Brunauer, Emmett, and Teller (BET) method with a Quantochrome Monosorb BET analyzer. Mercury porosimetry was used to determine total pore volume and macroporous surface area (20).

Biocarriers were analyzed by scanning electron microscopy with a Hitachi S570 electron microscope. Specimens were mounted on aluminum stubs and coated with gold-palladium alloy (60:40). Colonized biocarriers were fixed in formalin vapors for 24 h and air dried prior to analysis.

Biocarrier colonization and bioreactor configuration. Bioreactor studies were performed with 2.1-liter Kontes columns.

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The columns were packed with the various biocarriers and colonized by recycling cells through the matrices in a batch mode. *Pseudomonas* inocula were prepared by growing cells in 2.8-liter Fernbach flasks containing 750 ml of basal salts medium (BSM [5]) and 500 ppm of phenol. The preacclimated cell suspensions were pumped into the columns and recycled at a rate of 1.0 ml/min for 16 h. Phenol concentrations were monitored to preclude starvation conditions. After this procedure was repeated twice, the bioreactors received an influent feed of phenol-BSM.

Bioreactors were operated in an up-flow mode at 23°C. Columns were packed with biocarriers leaving a 200-ml headspace of medium above the biocarrier beds. The medium in the headspace was recycled to the base of the column at a flow rate 15 times higher than the influent feed flow rate. This improved mixing and oxygen distribution. Reactors received a common waste stream containing 1,000 ppm of phenol in BSM and were oxygenated with pure oxygen introduced in the recycle line prior to the influent feed. In most cases, 2 to 4 ppm of dissolved oxygen was maintained in the reactors. Influent and effluent samples were analyzed for contaminant concentration.

Chemical analysis. Degradation was monitored in the effluent by high-pressure liquid chromatography (HPLC) with a Perkin-Elmer HPLC. Separations were accomplished with a Supelco LC-18 column (150 mm by 4.6 mm) at a flow rate of 1 ml/min in an isocratic system containing 25% acetonitrile in buffer A (pH 3.5). Buffer A contained 3.14 g of *n*-heptanesulfonic acid, 2.0 ml of *o*-phosphoric acid, 2.0 ml of *n*-butylamine, and 0.5 g of ammonium acetate in a final volume of 4 liters of Milli-Q water. Detection was at 270 nm with a Perkin-Elmer LC-90 UV detector. Alternatively, degradation was measured by the chemical oxygen demand (COD) test, which measures the oxygen equivalent of the amount of organic matter oxidizable by potassium dichromate in 50% sulfuric acid. Solutions of influent and effluent were filtered through 0.45- μ m-pore-size filters, and 2 ml was added to tubes containing COD digestion reagent (Hach Chemical, Loveland, Colo.). The solutions were incubated at 150°C for 2 h, after which the COD (parts per million) was determined colorimetrically with a Hach DR/3000 spectrophotometer at 600 nm. In all cases, phenol mineralization as determined by HPLC analysis was within the experimental error of COD removal as determined by wet chemical analysis.

Colonization of biocarriers was determined by ATP analysis (4). Triplicate samples of biocarriers (two per analysis) were extracted with 200 μ l of Extralight (Analytical Luminescence Laboratory, San Diego, Calif.). Luminescence was determined with a Turner Designs model 20e luminometer after the addition of 100 μ l of Firelight (luciferin-luciferase reagent) to 100 μ l of extract. The average content of ATP was determined to be 3.65×10^{-10} μ g/CFU. This correlation of ATP to CFU was determined by measuring the ATP concentration and enumerating viable cell counts of suspensions of the microorganisms used to colonize the bioreactors. Alternatively, colonization of biocarriers was estimated by aseptically removing biocarriers from the bioreactors, crushing the beads with a mortar and pestle, and extracting the cells by suspending the crushed beads in 1 to 2 ml of phosphate-buffered saline. The solution containing cell suspensions and the residual crushed biocarrier were transferred to tubes and mixed extensively. The cell suspensions were diluted and plated onto solid media, and the number of CFU was determined after 48 h of incubation at 30°C.

Ammonia-N from influent and effluent media was measured with an Orion ammonia electrode (no. 95-12) standardized against an ammonium chloride standard.

TABLE 1. Physical properties of Type-Z biocarriers

Property	Value
Mean pore diam (μ m).....	7–10
Surface area (m^2/g).....	79 ^a
Accessible surface area (m^2/g) ^b	0.14–0.17
Total pore vol (ml/g).....	0.53
Accessible pore vol (ml/g) ^b	0.20–0.24
Crush strength (lb/in ²).....	20
Exchange capacity (meq/g).....	0.4

^a Determined by mercury porosimetry (20).

^b Determined on the basis of pore diameters >4 μ m.

Determination of the effect of system shocks on bioreactor performance. Once bioreactors reached steady-state kinetics for phenol degradation, they were subjected to the various system shocks that may be encountered during biotreatment. For these experiments, reactors were run at hydraulic retention times that resulted in ~80 to 95% degradation of influent phenol so that differences in bioreactor performances could be ascertained. (Hydraulic retention time is defined as the void volume of the reactor divided by the flow rate.)

(i) **Effect of acid and base shock.** Bioreactors were fed a common waste stream of phenol-BSM acidified to pH 2 with 18 mM sulfuric acid. After 24 h, the bioreactors received the standard medium. The effluents were monitored for pH and phenol degradation.

Similarly, bioreactors received a phenol waste feed at pH 12 for 72 h before being returned to the standard medium.

(ii) **Effect of oxygen deprivation.** Oxygenation of the bioreactors was discontinued for 9 days, with a constant flow of unoxygenated influent feed. Dissolved oxygen and phenol degradation levels were monitored.

(iii) **Effect of toxic shock loads.** The pseudomonads used for these studies were inhibited by cresol when the bacteria were grown in suspension culture on a shaker. Fortunately, *p*-cresol and phenol are easily resolved by HPLC, so that bioreactors could be subjected to toxic levels of *p*-cresol with subsequent monitoring of the effect of phenol degradation. Bioreactors received an 8,000-ppm spike of cresol injected at the influent feed line, and phenol degradation and cresol concentration in the effluent were monitored by HPLC.

(iv) **Effect of nutrient starvation conditions.** Bioreactors received a phenol feed devoid of exogenous nitrogen for a period of 16 days, after which the standard nutrient level was provided, and recovery of phenol degradative capabilities was determined.

RESULTS

Physical properties of Type-Z biocarriers. The physical properties of Type-Z biocarriers are summarized in Table 1. A scanning electron micrograph of an uncolonized biocarrier is shown in Fig. 1A. The matrix is composed of spherical clay particles that are bonded by an inorganic binder. The size of these particles can be modified to alter pore size. Furthermore, the inorganic oxide chemistry can be manipulated to change the surface properties. The biocarrier consists of a silica alumina matrix containing a zeolite molecular sieve. Zeolites are a class of microcrystalline aluminosilicate materials that are well known for their adsorptive, catalytic, and ion-exchange properties (1). The carrier comes in the form of 1/8- or 1/4-in. extruded pellets with a 1:1 aspect ratio such that a large fraction of the material porosity is in the macropore range. The matrix contains a high amount of surface area as determined

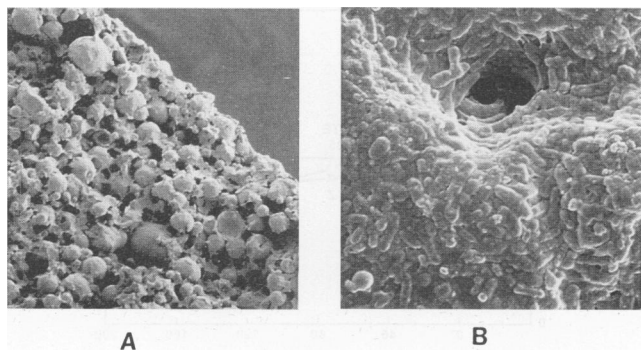


FIG. 1. Scanning electron micrographs of uncolonized (A) and colonized (B) Type-Z biocarriers.

by BET analysis ($130 \text{ m}^2/\text{g}$) and mercury porosimetry data ($77 \text{ m}^2/\text{g}$). However, these data can often be misleading because these measurements include the microporous surface area of the matrix that is not accessible to microorganisms (14). The surface area available for microbial colonization is 0.14 to $0.17 \text{ m}^2/\text{g}$, determined on the basis of pores with mean pore diameters of $\geq 4 \mu\text{m}$ (14, 17). By comparison, the accessible surface area reported for granular activated carbon is only $0.039 \text{ m}^2/\text{g}$ (Filtasorb 300 [14]). The total pore volume of this matrix, which is indicative of the number of cells that can be immobilized onto the biocarrier, is 0.5 ml/g . Evaluation of the mercury intrusion data indicated that $>60\%$ of the total pore volume is accessible to microorganisms (i.e., pore diameter of $>4 \mu\text{m}$).

Colonization of biocarriers. The microscopic appearance of a colonized biocarrier is shown in Fig. 1B. The aerobic pseudomonads used in this study extensively colonized the exterior of the biocarrier. Scanning electron microscopy analysis of the interior of the matrix revealed that the interior was not completely colonized but that colonization had occurred 0.5 to 1 mm from the external surface (data not shown). Microbial populations of 10^9 viable cells per g of biocarrier were measured by ATP analysis, and 9×10^8 CFU per g were obtained from standard plate count determinations. By using

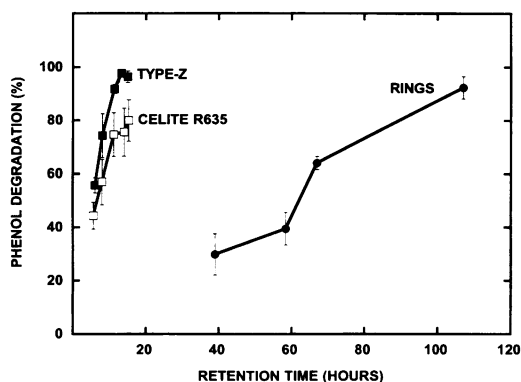


FIG. 2. Bioreactor efficiencies as a function of hydraulic retention time. Bioreactors containing various matrices for microbial immobilization were assessed for their abilities to degrade phenol at various hydraulic retention times as described in the text. Datum points represent the mean and standard deviation of 6 days of datum points for each retention time.

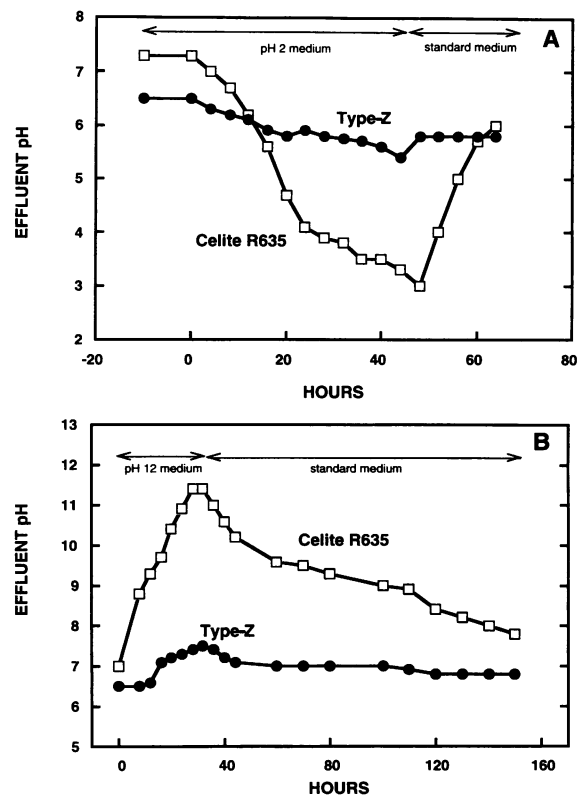


FIG. 3. Effect of acidic and basic pH media on bioreactors containing uncolonized Type-Z and Celite R635 biocarriers. Abiotic bioreactors received BSM either at pH 2.5 (A) or pH 12 (B) at a flow rate of 40 ml/h . Effluent pH was monitored as a function of time. Bioreactors were returned to the standard medium (pH 7.1) as designated in the figure.

the latter method, comparable colonization results were obtained for Celite R635.

Comparative degradation of phenol as a function of hydraulic retention time. The degradation of phenol was assessed with bioreactors containing various biocarriers colonized with *P. aeruginosa*. The bioreactors received a common waste stream of phenol ($1,000 \text{ ppm}$) and inorganic nutrients, and degradation was determined at various hydraulic retention times. Six days of datum points were collected for each retention time. A comparison of the degradative properties of bioreactors containing either Type-Z, Celite R635, or Flexiring biocarriers is shown in Fig. 2.

Expression of these data as a function of volumetric productivity (kilograms of phenol degraded per cubic meter per day) indicated that bioreactors containing microorganisms immobilized onto the Type-Z biocarrier are 4.5-fold more efficient than plastic packing and 1.3-fold better than the diatomaceous earth biocarrier Celite R635.

Resistance of immobilized microorganisms to fluctuation in system parameters. Immobilized pseudomonads were subjected to various system shocks typically encountered during full-scale biotreatment of aqueous wastes. As described before, the bioreactors received a common feed and the effects of the system aberrations were evaluated to determine bioreactor performance and the recovery of microbial populations.

Acid and base shocks. The ion-exchange properties of the zeolite-containing biocarrier are shown in Fig. 3. Abiotic

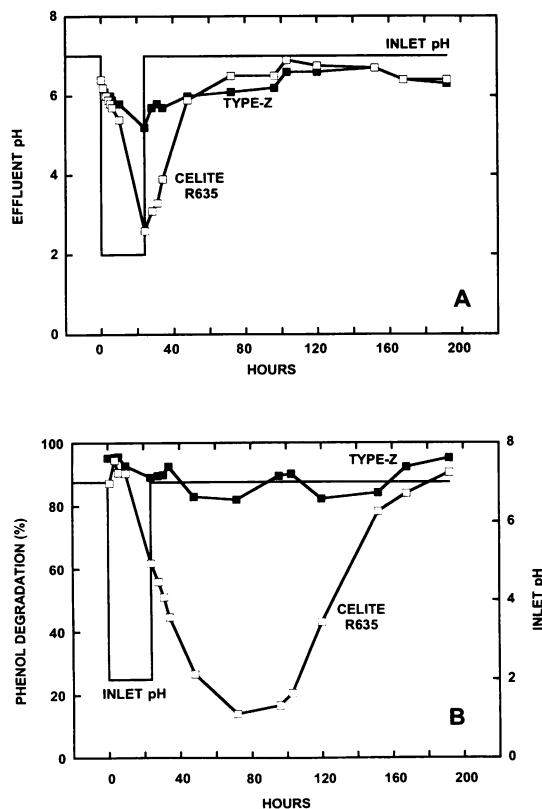


FIG. 4. Demonstration of acid shock resistance of bioreactors containing Type-Z biocarrier. Bioreactors were fed a common feed of phenol-BSM acidified to pH 2. The effluents were monitored for pH (A) and phenol degradation (B) as described in the text. The data are representative of nine independent experiments.

bioreactors were exposed to either acidic or basic BSM under normal operating procedures, and effluent pH was monitored as a function of time. The results demonstrated only moderate fluctuation in effluent pH from the bioreactor containing the Type-Z biocarrier. In contrast, the diatomaceous earth matrix Celite R635 did not provide any buffering capacity (Fig. 3).

The effect of a transient acid shock on the performance of bioreactors containing microorganisms colonized onto either the Type-Z or Celite R635 biocarrier is shown in Fig. 4. The effluent pH of the bioreactor containing Celite R635 declined to 2.5 after 2 column volumes of the acidified phenol feed (pH 2). This had a marked effect on bioreactor performance; phenol degradation fell to below 20%. Furthermore, the reactor did not fully recover until 5 days after normal operating conditions were reestablished. In contrast, the Type-Z bioreactor exhibited only a modest pH drop (~ 1 U) and bioreactor productivity remained constant (Fig. 4). Similar experiments have been performed with bioreactors containing pumice and plastic packing, and in all cases bioreactor productivities were significantly reduced (data not shown).

A bioreactor containing Type-Z biocarrier was not affected with respect to effluent pH or bioreactor productivity after receiving an inlet feed of pH 12. There was a slight increase in the effluent pH from the Celite R635 bioreactor, and this resulted in a corresponding decrease in phenol degradation (Fig. 5). Interestingly, the immobilized population prevented the pH in the Celite bioreactor from increasing to the levels that were observed with the abiotic reactor (Fig. 3B).

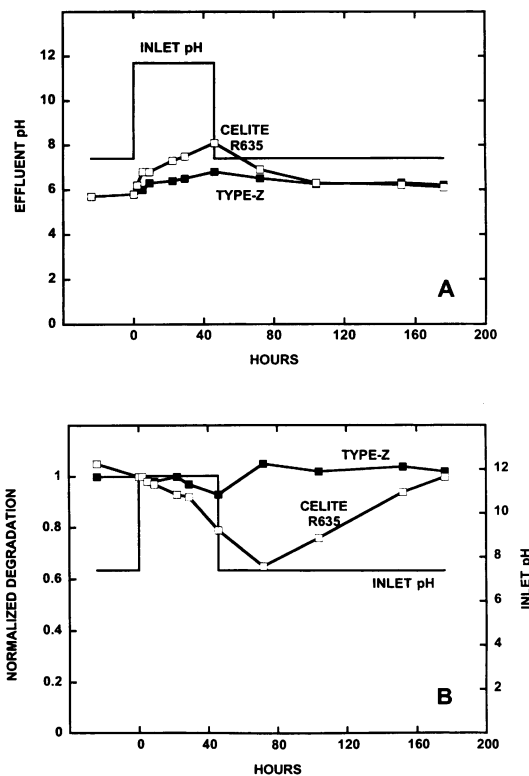


FIG. 5. Effect of base shock on bioreactors. Bioreactors containing Type-Z or Celite R635 biocarriers received a phenol-BSM feed at pH 12 for 48 h, after which the reactors received the standard medium (pH 7.2). (A) Effluent pH. (B) Normalized phenol degradation. The data are representative of duplicate independent experiments.

Oxygen deprivation. Oxygenation was discontinued in the bioreactor recycle loops, and phenol degradation was monitored. As shown in Fig. 6, the microorganisms immobilized onto either the Type-Z, Celite R635, or Flexirings biocarrier failed to degrade phenol without oxygen. After 9 days, oxygen

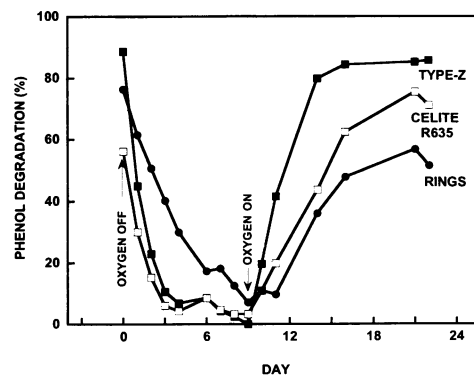


FIG. 6. Recovery of microorganisms immobilized onto Type-Z, Celite R635, and Flexirings biocarriers after oxygen deprivation. Oxygenation was discontinued from the bioreactors for 9 days and then resupplied, and recovery of phenol degradative capabilities was determined. The hydraulic retention time for the Type-Z and Celite R635 bioreactors was 14 h, whereas that for the Flexirings bioreactor was 78 h. The data are representative of four independent experiments.

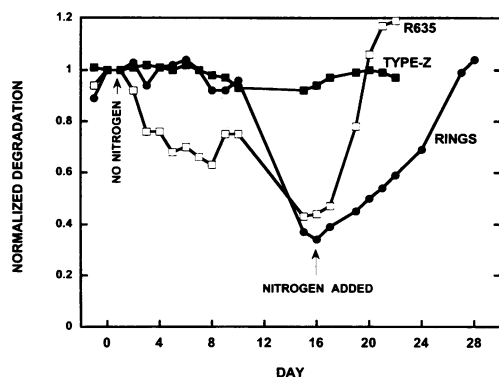


FIG. 7. Effect of nutrient limitation on bioreactor performance. Bioreactors received a phenol-salts feed (without nitrogen) for 16 days, after which the standard nutrient level was provided. Phenol degradative capabilities were determined as described in the text. Hydraulic retention times for the bioreactors were those given in the legend to Fig. 6. The data are representative of two independent experiments.

was returned to the bioreactors and bioreactor productivities returned to normal levels. However, reactor productivity recovered 24 h more rapidly with microorganisms colonizing the Type-Z biocarrier than with those colonizing the other surfaces.

Nutrient starvation. Bioreactors were subjected to nutrient limitation by excluding nitrogen from the influent waste stream. Microorganisms immobilized onto the Celite R635 biocarrier demonstrated a steady decline in bioreactor performance over a period of 16 days; addition of nitrogen to the feed resulted in rapid recovery of the system (Fig. 7). Similar effects were noted with the productivity of the bioreactor containing Flexirings. The decline in reactor performance, however, was delayed because of the difference in hydraulic retention times.

The Type-Z biocarrier binds ammonia, which serves as an indigenous source of nitrogen during this period. As shown in Fig. 7, there was no loss in the efficiency of phenol removal. Analysis of the effluent revealed that between 20 and 40 ppm of ammonia-N was bleeding from the system. Additional analysis of the effluent demonstrated that the amount of COD removed from the system was within 5% of the HPLC data, confirming that partial oxidation was not occurring.

Organic system shock. The bioreactors were subjected to a toxic organic surge of a 1/10th column volume of 8,000 ppm of *p*-cresol. The cresol was monitored in the effluent, as was the phenol biodegradation. The data shown in Fig. 8 demonstrate that each bioreactor experienced a loss in performance. For example, the phenol degradation was reduced 20% in the Type-Z bioreactor and 30% in the R635 bioreactor. Once cresol was diluted from the system (~40 h), the phenol degradative capabilities returned to normal.

DISCUSSION

The results of this investigation demonstrate that new biocarriers have been developed for microbial immobilization and use in bioreactor systems. The data indicate that these biocarriers support the dense colonization of microorganisms, improve volumetric productivities of bioreactors, and provide a surface that may increase the reliability of fixed-film biotreatment processes.

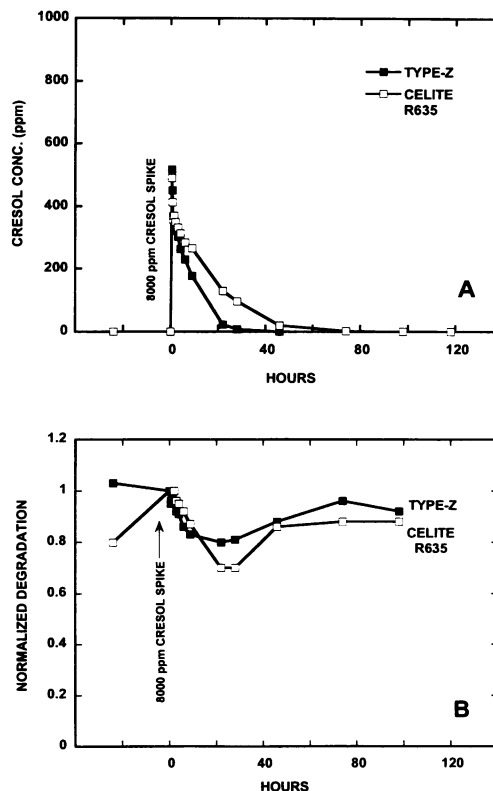


FIG. 8. Effect of cresol shock loads on bioreactors. Bioreactors colonized with bacteria degrading a 1,000-ppm phenol feed were subjected to 8,000 ppm of *p*-cresol as 1/10th column volume additions. The concentration of cresol in the effluent (A) and the degradation of phenol (B) were determined by HPLC.

The Type-Z biocarrier is a biocompatible, inorganic matrix that has a rough and porous structure (Fig. 1A). The rough surface properties facilitate microbial adhesion and adsorption during the immobilization process, whereas the pores, crevices, and irregularities of the biocarrier protect immobilized microorganisms from bioreactor shear forces (2, 3, 13). The high amount of surface area and total pore volume are conducive to the establishment and maintenance of luxuriant microbial populations (Table 1; Fig. 1B). The total accessible surface area available for microbial colonization is approximately four times that reported for activated carbon (14). The importance of accessible pore diameters and surface texture has been thoroughly documented by others (3, 12–15, 17, 18); high cell densities per unit volume result in higher volumetric productivity and faster throughput of wastes. A recent study was unable to distinguish between the performances of bioreactors containing nonporous materials, such as glass beads, and porous matrices (9). Our experience, as well as that of others (13, 14, 19), with nonporous, low-surface-area materials such as plastic or Isolite diatomaceous earth biocarriers does not support these findings. In comparative studies (Fig. 2), microorganisms immobilized onto the Type-Z biocarrier were shown to be considerably more proficient at degrading phenol than microorganisms immobilized onto plastic packing (four- to fivefold). Bioreactors containing the Type-Z biocarriers were only moderately more proficient (1.3-fold) than bioreactors containing the porous biocarriers (Celite R635).

Parameters that affect microbial metabolism include substrate concentrations, pH and E_h , temperature, dissolved oxygen, nutrients, and inhibitors. These factors must be controlled for effective full-scale biotreatment of wastes. Clearly, one of the advantages of immobilized-cell biotreatment processes over suspended-cell systems is that these conditions can be regulated more effectively with fixed-film bioreactors (9). Moreover, if system shocks do occur, immobilized cells exhibit reduced susceptibility, because the entire immobilized population is usually not killed and surviving microorganisms rapidly recolonize the biocarriers. This property was demonstrated in a recent study that showed the dependability of bioreactor systems during severe process upsets. The investigation by Heitkamp et al. (9) evaluated the loss and subsequent recovery of the degradative potential of immobilized populations after acid and heat shocks and desiccation and concluded that immobilized microorganisms display good recovery from these upset conditions regardless of the biocarrier surface texture. They furthermore reported that activated coconut carbon protected the microbial population from these process excursions.

Bioreactors containing the Type-Z matrices were more tolerant than the other bioreactors were to system excursions that reduce bioreactor utility by inhibiting or killing microbial populations. The dependability of these biocarriers can be ascribed to the ion-exchange properties of this zeolite matrix. For example, the abiotic control experiments shown in Fig. 3 clearly demonstrate the ion-exchange capability, and hence buffering properties, of the Type-Z biocarrier. During transient acid or base excursions of bioreactors containing colonized Type-Z biocarrier, the microbial population continued to degrade the influent waste contaminant (Fig. 4 and 5). Acid buffering capacity is due to the zeolite-mediated cation-exchange capability of the carrier, whereas the buffering during high-level base exposure was unexpected and is presumably due to the silicates in the ceramic structure of the matrix. As shown by Heitkamp et al. (9) and supported in this communication, other immobilization materials (e.g., diatomaceous earth, plastic packing, glass beads, and pumice) do not mediate against these pH shocks. Similarly, the ion-exchange properties of the zeolite-based Type-Z biocarrier are advantageous for use in bioreactor systems during periods of nutrient limitation. The zeolite-based matrix binds ammonia that can serve as a reserve source of nitrogen during these periods. As shown in Fig. 7, bioreactors containing the Type-Z product continued to degrade phenol when nitrogen was eliminated from the feed stream. In contrast, bioreactors containing Celite R635 or Flexirings exhibited a steady decline in reactor performance and recovered only when exogenous nitrogen was supplied to the feed.

The resilience of microorganisms immobilized onto biocarriers is shown in Fig. 6 and 8. For example, during oxygen deprivation, phenol degradation was diminished because metabolism of this substrate by *Pseudomonas* is oxygen-dependent. However, once the system was recharged with oxygen, the population readily responded and phenol degradation returned to steady state (Fig. 6). In a separate experiment, microorganisms exhibited a similar recovery from a toxic shock with cresol. The *Pseudomonas* strain used in this study is sensitive to high levels of cresol. As demonstrated in Fig. 8, these immobilized bacteria recovered completely after the cresol was diluted from the system. Toxic surges of organic chemicals represent the most common and detrimental process shock encountered during full-scale biotreatment (6, 22). Currently, we have under development an improved Type-Z

biocarrier with adsorptive properties that can mitigate process upsets due to organic shock loads.

In summary, a new biocarrier has been developed for use in fixed-film biotreatment processes. The available data indicate that this biocarrier meets all of the criteria for providing a suitable medium for microbial colonization (12, 15, 17). In addition, microorganisms immobilized onto this biocarrier exhibit superior bioreactor performance. Finally, this matrix maintains an environment that permits continuous microbial metabolism of environmental contaminants during certain periods of process shocks and may represent an enabling technology for the full-scale biotreatment of aqueous wastes.

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